DIVERSITY OF STAPHYLOCOCCUS COAGULASE- POSITIVE AND NEGATIVE STRAINS OF COALHO CHEESE AND DETECTION OF ENTEROTOXIN ENCODING GENES

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Three hundred samples of coalho cheese, from 15 different brands, seven handmade and eight industrialized, were evaluated in relation to the contamination profile by *Staphylococcus* coagulase-positive and negative and the occurrence of staphylococcal enterotoxin encoding genes. Two hundred and eight isolates of *Staphylococcus* sp. were subjected to phenotypic identification and 95 were subjected to genotypic identification through *femA* gene research and detection of genes (*sea, seb, sec, sed, see, seg, seh, sei and sej*) encoding enterotoxins, using the polymerase chain reaction technique (PCR). A total of 14 species of *Staphylococcus* were identified, of which three were coagulase-positive and eleven negative, especially: *S. aureus*, *S. xylosus*, *S. cohnni* spp. *cohnii*, *S. saprophyticus*, *S. epidermidis*, *S. hyicus*, *S. lentus*, *S. sciuri*, *S. cohnii* spp. *urealyticus*, *S. haemolyticus*, *S. chromogenes*, *S. lugdunensis*, *S. hominis* e *S. intermedius*. In all the samples of handmade coalho cheese there was a prevalence of S. *aureus*; while at industrial samples *S. xylosus* (87.5%) and *S. cohnii spp cohnii* (50%) were predominant. The presence of the *femA* gene was detected in 95% (38/40) isolates of positive *Staphylococcus* coagulase and 16.4% (9/55) of coagulase-negative isolates. Among the enterotoxin encoding genes evaluated, there was prevalence of the *seh* gene (53.2%) in coagulase-positive strains and of the *sec* gene (46.8%) in coagulase-negative strains. The results suggest a re-evaluation of the Brazilian microbiological standards in relation to the genus *Staphylococcus* in foods.

KEYWORDS: FOOD POISONING; PCR; DAIRY PRODUCTS.; STAPHYLOCOCCAL ENTEROTOXINS.

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1 INTRODUCTION

Staphylococcus sp. is one of the pathogenic agents most commonly involved in outbreaks of food poisoning. The peculiarities of its habitat makes its presence widely distributed in nature, being transmitted by food handlers (mostly asymptomatic carriers)and animals, especially dairy cows with mastitis.

Among dairy products, cheeses have been one of the products most often involved in cases of food poisoning, and in the Northeast the emphasis is on coalho cheese. Throughout the region, the production of coalho cheese can be divided into two sectors: the medium-sized companies supervised and regulated by official organizations, and small handmade units, mainly located in rural areas who have nonesupervision (Dantas *et al.,* 2013).

Cheese, especially the handmade kind, drawn mostly from raw milk and, in most cases, poor hygienic conditions, has been considered a source of food-borne pathogens. Therefore, the microbiological safety of the product is of great importance to the health of consumers because of the risk of causing food borne illness.

The level of contamination by coalho cheese because of pathogenic bacteria, especially the *Staphylococcus* genus, have been high (Tigre; Borelly, 2011; Oliveira *et al.,* 2015; Evangelista-Barreto *et al.*, 2016; Vieira, 2017). These bacteria, when presented in high populations (10^5-10^6 UFC mL^{-1} or g^{-1}) and under appropriate conditions (temperature, pH, water activity and O_2), represents a public health problem because of the ability to produce enterotoxins and cause staphylococcal food poisoning.

Although *S. aureus* is the principal and most studied representative of the genus, the presence and expression of enterotoxins and other virulence factors can be observed in other species of staphylococci, such as coagulase negative staphylococci (Mazzariol *et al.,* 2012; Ünal, Çinar, 2012)

Recently, the list of toxins expanded thanks to the discovery of new genes: 20 distinct types of enterotoxins have been identified so far, however all are similar in structure and sequence. The classical (SEA, SEB, SEC_1, SEC_2, SEC_3, SED e SEE) are the most frequent, and thirteen other enterotoxins (SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER E SEU) were identified and their genes (*seg, seh, sei, sej*, *sek, sel, sem, sen, seo, sep, seq, ser and seu*) correspondingly described.

The research of genes coding for toxins, through traditional identification and molecular techniques such as the PCR technique and Multiplex PCR, has been a useful tool in the differentiation of *Staphylococcus aureus* and is therefore a quick and safe alternative for bacteria identification. These methodologies guarantee reliability in the results, besides contributing to minimize the occurrence of foodborne diseases.

Thus, the objective of this study was to identify species of *Staphylococcus* coagulase-positive and negative strains of coalho cheese and evaluate the occurrence of staphylococcal enterotoxin encoding genes.

2 MATERIALS AND METHODS

2.1 ISOLATION AND PHENOTYPIC IDENTIFICATION OF STAPHYLOCOCCUS SPP

Staphylococcus spp. was isolated from 300 samples of coalho cheese, from 15 different brands (seven handmade and eight industrial), collected weekly in retail stores in Fortaleza, Brazil from July 2016 to June 2017. The isolation was done in Baird-Parker agar (Merck), according to the methodology described in the literature (Bannerman, 2003, Bennett; Lancette, 2001). After prior screening with conventional biochemical tests (catalase production, coagulase, sensitivity and lisostaphin, fermentation of manitol and glucose and DNAse), 208 isolates, 162 being coagulase-positive and 46 coagulase-negative, phenotype were phenotypically characterized at the species level

by using the *Staphylococcus* and *Micrococcus* identification system (BioMérieux SA, Marcy-l'Etoile - France). *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 14990 were used as reference strains.

2.2 DNA EXTRACTION

The strains of *Staphylococcus* sp. had their genetic material extracted according to the protocol described by Rosec and Gigaud (2002), with some changes proposed by Ângelo *et al.* (2007). Briefly, 2 mL of the culture were transferred to an Eppendorf tube and centrifuged (High Speed Refrigerated Micro Centrifuge; VS - 15000CFNII) at 12.000 *g* for 10 minutes. The supernatant was discarded and the cells were re-suspended in 500 µL TE buffer [Tris-HCl 10mM pH 8.0 e EDTA 1 mM pH 8.0] and centrifuged at 12.000 *g* for 10 minutes. Once the supernatant was again discarded, the cells re-suspended in 200 µL TE buffer, and 15 mL of 1mg/mL lisostaphin was added to it (Sigma, L7386), mixed (vortex shaker) and incubated at 37 °C for 30 minutes. Then 10 µL of K 20 mg/mL proteinase (Sigma, P6556) were added and the cells were incubated at 60 °C for 20 minutes and then at 100 °C for 10 minutes. The total DNA obtained was frozen at -20 °C until PCR amplification.

2.3 POLYMERASE CHAIN REACTION

The PCR reaction was performed according to methodology described in the literature (Ângelo et al., 2007; Rosec and Gigaud, 2002) using known (oligonucleotide) primers (Table 1). The amplification was performed in a thermo cycler (Techne; TC 512) and contained a mixture of 5 µL of PCR 1X (Invitrogen) buffer, 1.0 mM $MgCl_2$ (Invitrogen), 0.2 mM of each dNTP, 0.2 µM of each primer (Alpha DNA) and 1.0 U Taq DNA Polymerase Recombinant (Invitrogen). 5 µL of extracted DNA were added to the mixture and the volume was completed up to 50 µL RNAse-free and DNAse-free (Gibco) distilled water. The amplification conditions were as follows: heating at 94 °C for three minutes, followed by 35 cycles of amplification (denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 30 seconds), final extension at 72 °C for ten minutes and maintenance of the samples at 4 °C until the gel application. The amplified DNA fragments were visualized in agarose gel 1.5% (p/v) (Amersham Biosciences) stained with ethidium bromide solution 0.005% (p/v) and photographed in documentary photos (Canon; Power Shot A620).

2.4 RESEARCH OF THE FEMA GENE IN STRAINS OF STAPHYLOCOCCUS AUREUS

Forty strains of *Staphylococcus aureus*, composed of 20 isolated strains from handmade coalho cheese and 20 industrialized coalho cheese strains, identified based on conventional biochemical tests and phenotypic tests, were subjected to genotypic confirmation by the amplification of a fragment of 132 pb for the *femA* gene. The *S. aureus* ATCC 25923 strain was used as the positive control for the reaction.

2.5 DETECTION OF ENTEROTOXIN ENCODING GENES

The research of the staphylococcal enterotoxins encoding genes (sea, seb, sec, sed, see, seg, seh, sei, sej, sel) was performed in 95 strains of *Staphylococcus*, including coagulase-positive (40) and negative (55), selected among those previously identified by phenotypic tests, through the PCR and Multiplex PCR. In the Multiplex PCR, four conformations were used (*sea*+*sed*), (*seb*+*sel*), (*sec*+*see*); (*seh*+*sei*). Simple PCR was used for single genes *seg* and *sej*. *S.aureus* 95-4776B for *sea*; *S. aureus* 91-2415D for *seb*, *S. aureus* ATCC 19095 for *sec, seh, sei, seg* and *sel*; *S. aureus* ATCC 23235 for *sed, seg, sei* and *sej*; *S. aureus* ATCC 27664 for see was used as positive controls for the reaction.

TABLE 1. OLIGONUCLEOTIDE USED IN THE PCR REACTIONS FOR THE AMPLIFICATION OF THE SPECIFIC GENE FOR THE *STAPHYLOCOCCUS* SPECIES AND DETECTION OF THE *STAPHYLOCOCCI* ENTEROTOXIN ENCODERS.

Oligonucleotide (5'– 3')	Gene(s)	Amplified Product	Reference
SEA_1: ACG ATC AAT TTT TAC AGC SEA_2: TGC ATG TTT TCA GAG TTA ATC	*sea*	544 pb	Betley; Mekalanos (1988)
SEB_1: GAA TGA TAT TAA TTC GCA TC SEB_2: TCT TTG TCG TAA GAT AAA CTT C	*seb*	416 pb	Jones; Khan (1986)
SEC_1: GAC ATA AAA GCT AGG AAT TT SEC_2: AAA TCG GAT TAA CAT TAT CCA	*sec*	257 pb	Bohach; Schievert (1987)
SED_1: TTA CTA GTT TGG TAA TAT CTC CTT SED_2: CCA CCA TAA CAA TTA ATG C	*sed*	334 pb	Bayles; Iandolo (1989)
SEE_1: ATA GAT AAA GTT AAA ACA AGC AA SEE_2: TAA CTT ACC GTG GAC CC	*see*	170 pb	Couch et al. (1988)
SEG_1: ACG TCT CCA CCT GTT GAA GG SEG_2: TGA GCC AGT GTC TTG CTT TG	*seg*	400 pb	Munson et. al (1998)
SEH_1: TCA CAT CAT ATG CGA AAG CAG SEH_2: TAG CAC CAA TCA CCC TTT CC	*seh*	357 pb	Ren et al. (1994)
SEI_1: TGG AAC AGG ACA AGC TGA AA SEI_2: TAA AGT GGC CCC TCC ATA CA	*sei*	467 pb	Munson et al. (1998)
SEJ_1: CAG CGA TAG CAA AAA TGA AAC A SEJ_2: TCT AGC GGA ACA ACA GTT CTG A	*sej*	426 pb	Zhang et al. (1998)
SEL_R: CTG TTT GAT GCT TGC CAT TG SEL_F: CAC CAG AAT CAC ACC GCT TA	*sel*	240 pb	Cremonesi et al. (2006)
$FEMA_1$: AAA AAA GCA CAT AAC AAG CG $FEMA_2$: GAT AAA GAA GAA ACC AGC AG	*femA*	132 pb	Mehrotra et al. (2000)

3 RESULTS AND DISCUSSION

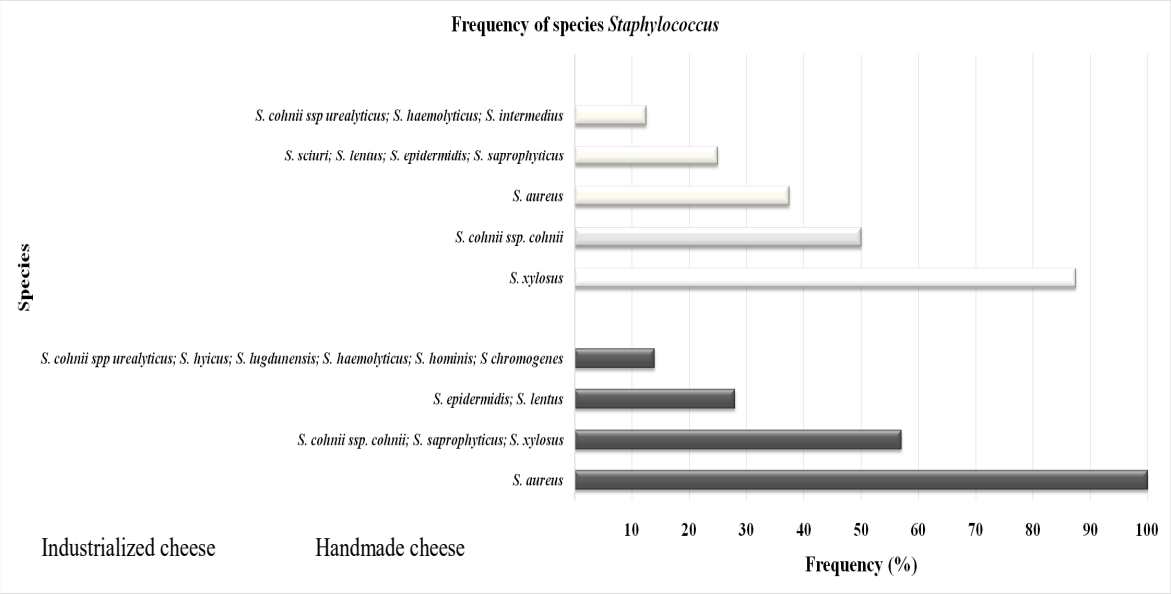
The coalho cheese samples showed a high population of *Staphylococcus* sp. The counts ranged from 1.3 x 10^6 UFC/g to 6.6 x 10^9 UFC/g in handmade cheeses and 1.3 x 10^7 UFC/g to 2.9 x 10^{10} UFC/g in industrialized cheese. This level of contamination by *Staphylococcus* sp. is considered high and may facilitate the production of staphylococcal enterotoxins under suitable environmental conditions.

Astudy carried out on samples of coalho cheese sold in the district of Cabo de Santo Agostinho, PE, Brazil, Oliveira *et al*. (2010) observed the incidence of *Staphylococcus* sp. in 76.2% of the samples, whose counts ranged from 1.6 x 10^3 to 2.0 x 10^5 UFC/g. Similar results were found by Silva Junior (2017), who evaluated samples of coalho cheese sold in Paraiba, Brazil, and found high populations of *Staphylococcus* sp. (1,1 x 10^5 UFC/g) in 100% of the samples.

Among the 300 samples analyzed, 327 isolated types of *Staphylococcus* were obtained, 145 of them being coagulase-negative and 182 of them coagulase-positive. After preliminary screening based on conventional biochemical tests, 208 isolated characteristics of the genus were selected. Phenotypic characterization made the identification of 193 isolates (117 of handmade cheeses and 76 industrialized cheeses) possible distributed in 14 species of *Staphylococcus*, three being coagulase-positive and 11 being coagulase-negative.

There was a prevalence of the species *S. aureus* (106/193), *S. xylosus* (40/193), *S. cohnni* ssp. *cohnii* (17/193), *S. saprophyticus* (6/193), *S. epidermidis* (4/193), *S. hyicus* (4/193), *S. lentus* (4/193), *S. sciuri* (4/193), *S. cohnii spp. urealyticus* (2/193), *S. haemolyticus* (2/193), *S. chromogenes* (1/193), *S. lugdunensis* (1/193), *S. hominis* (1/193) and *S. intermedius* (1/193). Among these species, a high frequency of *S. aureus* (100%) was found in samples of handmade coalho cheese and *S. xylosus* (87.5%) and *S. cohnii* ssp. *cohnii* (50%) in samples of industrialized coalho cheese (Figure 1).

FIGURE 1. FREQUENCY OF THE *STAPHYLOCOCCUS* SPECIES, IDENTIFIED THROUGH THE API®-STAPH SYSTEM, IN THE HANDMADE AND INDUSTRIALIZED COALHO CHEESE.



The high frequency of *S. aureus* (100%) in samples of handmade coalho cheese can be associated to contamination of raw milk, re-contamination after pasteurization, inadequate storage conditions and the handler, which may favor the dissemination of this bacterium in food. It is important to highlight that *S. aureus* has stood out as the main species responsible for the cases of staphylococcal food poisoning outbreaks due to the possibility of producing staphylococcal enterotoxins.

Study carried out by Andrade *et al.* (2011), demonstrated that *Staphylococcus* species prevalent in samples of coalho cheese were: *S. aureus* (100%), *S. xylosus* (87,5%) and *S. cohnii* subsp. *cohnii* (50%).

In another study, Vieira (2017) evaluated 179 isolates obtained at the colonial cheese sold in Porto Alegre, Brazil, and found the following species in 33% of the samples: *Staphylococcus equorum* (10), *S. vitulinus* (6), *S. hyicus* (4), *S. saprophyticus* (4), *S. epidermidis* (3), *S. carnosus* (1), *S. carnosus subsp. carnosus* (1), *S. capitis* (1), *S. chromogenes* (1), *S. fleurettii* (1), *S. haemolyticus* (1), *S. succinus subsp. casei* (1) and *S. warneri* (1).

The high frequency of strains of coagulase-negative staphylococci such as *S. xylosus, S.cohnii ssp. cohnii, S. saprophyticus, S. epidermidis and S. lentus* detected in samples of coalho cheese, demonstrates the need of a re-evaluation of the microbiological standards established by Brazilian legislation.

In 40 isolates of *S. aureus,* evaluated by PCR, the amplification of the fragment by 132 bp was found, specifically for the *femA* gene, in 95% (38/40) of the strains tested. This result demonstrates greater specificity and discriminatory power of genetic analysis.

In a similar study, Silva (2015) evaluated strains of S. *aureus* coagulase positive and negative isolated from cheese and detected the presence of the *femA* gene in 97,5% (39/40) of the isolates tested. Hassan *et al*. (2008) evaluated three molecular markers, among these the *femA*, for the detection of *S. aureus* and observed in the 45 strains tested the gene amplification.

The presence of enterotoxin encoding genes were observed in 49.5% (47/95) of the strains tested (Table 2). Among the 47 strains with SEG and SEH enterotoxin encoding genes SEG and SEH, it was found a prevalence of the seh gene in 53.2% (25/47) of the isolates, nine of which were handmade and 16 of which were industrialized.

TABLE 2. RESEARCH OF THE *STAPHYLOCOCCI* ENTEROTOXIN ENCODING GENES IN STRAINS OF *STAPHYLOCOCCUS* ISOLATED FROM HANDMADE AND INDUSTRIALIZED COALHO CHEESE SAMPLES

Cheese	Quantity of Strains	Coagulase	Amplified Genes
Handmade	20	+	*seg* (2); *seh* (5)
	25	-	*seg* (9); *seh* (4)
Industrialized	20	+	*seg* (2); *seh* (15)
	30	-	*seg* (9); *seh* (1)
Total strains evaluated	**95**		**47**

* Values in parenthesis indicate the quantity of strains that amplified one of the genes.

The gene *seg* was detected in 46.8% (22/47) of the isolates, 11 in each type of cheese. The genes encoding enterotoxin SEA, SEB, SEC, SED, SEE, SEI, SEJ and SEL were not detected in the isolates studied. These results indicate that the occurrence of the *Staphylococcus* spp enterotoxin encoding genes isolated from coalho cheese, was not very diverse.

Among the species with coagulase-negative and enterotoxigenic potential that will amplify a fragment for the *seg* and *seh* genes, we can highlight: *S. cohnii* ssp. *cohnii*, *S. cohnii* spp. *urealyticus*, *S. chromogenes, S. epidermidis*, *S. hominis, S. hyicus, S. lentus, S. lugdunensis, S. saprophyticus* and *S. xylosus* (Table 3). The presence of enterotoxigenic *Staphylococcus* species in samples of coalho cheese, both handmade and industrialized, may represent a potential danger to health because of the potential of causing staphylococcal food poisoning to consumers.

Rall *et al.* (2008) detected genes (*sea, seb, sec, sed, see, seg, seh, sei and sej)* encoders of staphylococcal enterotoxins in staphylococcal 57 strains isolated from raw and pasteurized milk and found the prevalence of genes *seg* (11 strains), followed by *sei* (10 strains) and *seh* and *sej* (3 strains each). In another study, Acosta *et al*. (2017) researched the genes (*sea, seb, sed, seg, seh* and *sei*) encoders of enterotoxins in strains of S*. aureus,* isolated from milk, and observed the presence at last one of these genes in 48,1% (13/27) of the strains studied and the frequency of genes *sea* was 33.3%, *seh* 18.5%, *sei* 11.1% and s*ed* 7.4%.

Freitas *et al*. (2009) researched staphylococcal enterotoxin encoding genes in strains of *Staphylococcus* coagulase-positive and negative, isolated from coalho cheese, and detected the presence of the following genes: *tst* (1/18), *sec* (2/18), *sed* (2/18), *seg* (4/18), *seh* (3/18), *sei* (4/18) and *sej* (2/18) in 90% (18/20) of the strains. Morandi *et al.* (2009) also investigated enterotoxin encoding genes (*sea, sec, sed, seg, seh, sei, sej and sel*) in 122 strains of *Staphylococcus* spp., isolated from dairy products and found the presence of the genes *sea* (13), *sed* (3), *seg* (1), *seh* (3) and *sei* (1) in 20 coagulase-positive strains.

Kérouanton *et al*. (2007) evaluated the potential of enterotoxigenic strains of *S. aureus* associated with outbreaks of staphylococcal food poisoning in France and found the presence of one or more of the genes studied in 29 strains. There was the prevalence of the gene *sea* (23/29) and *sed* (12/29). In another study, Luz (2009) found in strains of *Staphylococcus aureus*, isolated from milk and coalho cheese, the presence of the genes *seg, seh, sei and sej* in 93.6% of strains evaluated.

These results indicate that enterotoxigenic strains of *Staphylococcus* found in foods require greater attention because they put food security in danger. However, the high frequency of enterotoxin encoding genes, from *Staphylococcus* coagulase-positive and negative strains, detected in samples of coalho cheese, represent the risk of it causing staphylococcal food poisoning to consumers.

TABLE 3. OCCURRENCE OF GENES *SEG* E *SEH* IN *STAPHYLOCOCCUS* SPP. ISOLATED FROM DIFFERENT SAMPLES OF HANDMADE AND INDUSTRIALIZED COALHO CHEESE.

Cheese	Brand	Species	Amplified Genes
Handmade	A	*S. chromogenes (1)*, S. epidermidis (1), S. saprohyticus (1)*	3 *seh*
	B	*S. aureus (1)*	1 *seg*
	C	*S. cohnii* ssp *urealyticus (1), S. hominis (1), S. saprohyticus (1)*	3 *she*
	D	*S. cohnii ssp cohnii (2), S. lentus (1), S. saprophyticus (1), S. xylosus (1)*	5 *seg*
		S. aureus (1)	1 *she*
	E	*S. cohnii ssp cohnii (1), S. epidermidis (1), S. lugdunensis (1)*	3 *seg*
		S. aureus (1)	1 *she*
	F	*S. aureus (1), S. hyicus (1)*	2 *seg*
		S. aureus (1)	1 *she*
Industrialized	H	*S. xylosus (4)*	4 *seg*
	I	*S. xylosus (3), S. lentus (2)*	5 *seg*
		S. xylosus (1)	1 *she*
	J	*S. lentus (1)*	1 *seg*
		S. cohnii spp cohnii (1)	1 *she*
	L	*S. aureus (2)*	1 *seg*; 1 *she*
	N	*S. aureus (3)*	3 *she*
	O	*S. aureus (10)*	10 *she*

* Values in parenthesis indicate a quantity of strains of the species that amplified a fragment for the gene.

CONCLUSIONS

- The diversity of the species of coagulase-positive and negative present in coalho cheese is represented by *S. aureus*, *S. xylosus*, *S. cohnni* ssp. *cohnii*, *S. saprophyticus*, *S. epidermidis*, *S. hyicus*, *S. lentus*, *S. sciuri*, *S. cohnii* spp. *urealyticus*, *S. haemolyticus*, *S. chromogenes*, *S. lugdunensis*, *S. hominis* and *S. intermedius.*
- There is a high frequency of *Staphylococcus aureus* in handmade coalho cheese. As for the industrialized cheese, there is a higher frequency of coagulase-negative species, with predominance of *S. xylosus, S. cohnni ssp. cohnii.*
- The genotypic identification (PCR) confirmed 95% of the strains of *S. aureus* identified by biochemical tests. The genes *seg* and *seh*, encoders of the new enterotoxins (SEG and SEH), occurs in *Staphylococcus* species isolated from coalho cheese.
- The gene *seg* was predominant in the coagulase-negative species such as *S. cohnni* spp. *cohnii, S. cohnni* ssp. *urealyticus, S. chromogenes, S. epidermidis, S. hominis, S. hyicus, S. lentus, S. lugdunenis, S. saprophyticus and S. xylosus*. While the *seh* gene was predominant in *S. aureus.*

- The high frequency of coagulase-negative staphylococcal strains detected in samples of coalho cheese, suggests the need for a re-evaluation of the microbiological standards established by Brazilian legislation for cheeses that refer only to coagulase- positive *Staphylococcus*.

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